African Journal of Microbiology Research Vol. 4(24) pp. 2754-2762, 18 December, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2010 Academic Journals

Full Length Research Paper

Expression and identification of a small recombinant beefy meaty peptide secreted by the methylotrophic yeast *Pichia pastoris*

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Accepted 10 November, 2010

A tasty peptide, beefy meaty peptide (BMP), which was initially isolated from beef digested by papain, has potential as a new flavor enhancer. BMP is a small peptide consisting of eight amino acids. In this study, DNA encoding BMP was synthesized and cloned into the expression vector pPICZαA to obtain the recombinant expression vector pPICZαA-BMP. The recombinant vector was linearized and then integrated into the genome of *Pichia pastoris* strain X-33 by electroporation. The recombinant strain *P.p*2 was then incubated in BMGY medium and then induced in BSM medium for expression of BMP. The fermentation broth was centrifuged and the supernatant was purified by gel-filtration chromatography. Purified recombinant BMP was then tested by high performance liquid chromatography-mass spectrometry (HPLC-MS). After optimization of the culturing process, the yield of BMP reached 10 mg/L in the clarified broth. The results of mass spectrometry/mass spectrometry (MS/MS) showed that recombinant BMP secreted from *P. pastoris* had a molecular weight of 873.7 Da rather than the expected 847 Da. We speculate that the last amino acid, Ala, of the BMP sequence was replaced with Pro in the BMP fragment.

Key words: beefy meaty peptide, *Pichia pastoris*, peptide expression, HPLC-MS.

INTRODUCTION

Beefy meaty peptide (BMP) is a small peptide consisting of eight amino acids that was initially extracted from beef digested with papain and has been described as having a 'strong delicious taste' (Yamasaki and Maekawa, 1978). Its primary structure was proposed to be: H-Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala-OH (K-G-D-E-E-S-L-A) and this sequence was verified by synthesis of this compound (Yamasaki and Maekawa, 1980). The molecular weight of BMP is about 847 Da. Synergisms of BMP with salt and monosodium glutamates (MSG) have been reported (Wang et al., 1996). BMP is a heat-stable peptide and can therefore be for heat-treated for use in the food industry (Wang et al., 1995). BMP is therefore a novel natural flavor enhancer, similar to MSG, with extensive commercial potential.

Specific tasty peptides are generally synthesized either chemically or enzymatically. However, neither strategy is feasible for large-scale, commercial production of tasty peptides due to the high production costs (Muheim et al.,

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Abbreviations: Mut, methanol utilization; **Mut$^+$,** methanol utilization plus; **Mut^S,** methanol utilization slow; **MD,** minimal medium containing dextrose; **MM,** minimal medium containing methanol; **YPD,** yeast extract-peptone-dextrose medium; **BMGY,** buffered glycerol-complex medium; **BMMY,** buffered methanol-complex medium; **BSM,** basal salts medium; **PCR,** polymerase chain reaction; **AOX,** gene encoding alcohol oxidase.

Table 1. Plasmids and strains used in this study.

Plasmid or strain	Description	Source
pPICZαA	Expression Vector used to clone cDNA, Zoe^R, P_{AOX1}, α-factor signal sequence	Invitrogen
pPICZαA-BMP	Zoe^R, P_{AOX1}, α-factor signal sequence, BMP gene	This study
P. pastoris X-33	Expression host for BMP expression, wild type, Mut^+ phenotype, His^-, promoter of *AOX*1	Invitrogen
P. pastoris X-33/ pPICZαA	Integrated with pPICZαA, used as negative control for BMP expression	This study
P. pastoris X-33/ pPICZαA-BMP	*P. pastoris* X-33 integrated with pPICZαA-BMP, Mut^- phenotype	This study
E. coli DH5α	Host for amplification of plasmid pPICZαA	Our lab

1998). With advances in biotechnology, recombinant DNA technology has more recently been used in the flavor industry (Muheim et al., 1998; Lerch et al., 1997; Bolen et al., 2003). The successful production of some small tasty recombinant peptides, such as BMP, cheese tasty peptide and bitter peptide, in *Escherichia coli* or *Saccharomyces cerevisiae* host cells encouraged us to consider the application of recombinant DNA techniques to produce BMP in *Pichia pastoris*. Our objective was both to over express this small peptide and to produce the peptide as efficiently as possible to minimize the cost of the product.

In this work, we attempted to produce BMP in *P. pastoris* using recombinant DNA technology. The gene encoding BMP was synthesized and cloned into the plasmid vector, pPICZαA, and the recombinant strain *P. pastoris*-BMP was constructed. The optimal fermentation conditions for the recombinant strain *P. pastoris*-BMP in shaking flask cultures were investigated and the target recombinant BMP was identified.

MATERIALS AND METHODS

Strains and plasmids

E. coli strain DH5α was used as the host strain for plasmid amplification and the plasmid, pPICZαA (Table 1), was used as a vector to integrate the target BMP gene into the methylotrophic yeast *P. pastoris* host strain X-33 (Table 1). The design and synthesis of the target gene of BMP and the construction of the expression vector and strain were performed using standard protocols (according to the Invitrogen manual, 2010).

Enzymes, reagents and culture media

Restriction endonucleases and T4 DNA ligase were purchased from Takara Biotechnology Dalian Co. Ltd. (Dalian, China). The BMP gene containing restriction sites and BMP (95% purity, used as a standard sample) were commercially synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). All other chemicals used were of analytical grade. The yeast culture media, MM, MD, YPD, BMGY, BMMY and BSM, were prepared according to the manufacturer's guidelines (Invitrogen, 2010).

Construction of the expression plasmid

In this study, the secretory mode of expression was employed for BMP. The BMP gene was PCR amplified using the primers: forward 5′-TCGAAAAGCGCAAGGGCGATGAGGAATCACTGGC-3′ and reverse 5′-CTAGGCCAGTGATTCCTCATCGCCCTTGCGCTTT-3′. After *Xho*I/*Xba*I digestion of the PCR product, the BMP gene was cloned into the pPICZαA vector at the *Xho*I (5′ end) and *Xba*I (3′ end) restriction sites to generate the plasmid pPICZaA-BMP. In pPICZaA-BMP, the BMP gene was under the control of the AOX1 promoter and in-frame with the α-factor signal sequence, the most commonly used secretion signal from *S. cerevisiae*. Proper construction of the final vector, pPICZαA-BMP, was verified by restriction analysis and DNA sequencing (Koganesawa et al., 2002).

Electroporation of *P. pastoris*

Transformation of *P. pastoris* strain X-33 was achieved by electroporation with *Pme*I-linearized pPICZαA-BMP and pPICZαA (negative control) plasmids. Screening for yeast strains containing the pPICZαA-BMP plasmid was performed as described in the Invitrogen manual.

Determination of the methanol utilization (Mut) phenotype

Using sterile toothpicks, 12 transformants were picked and patched onto MM and MD plates, respectively. The plates were then incubated for two days at 28℃ and the sizes of the resulting single colonies were compared.

Fermentation of *P. pastoris*-BMP

BMMY medium contains yeast extract and peptides, resulting in complex background proteins or peptides at the end of fermentation. For this reason, BSM medium was selected as the inducing medium instead of BMMY medium in this study to reduce the level of background proteins or peptides in the fermentation broth.

In the growth phase, transformed yeast cells (strain X-33 transformed with pPICZaA-BMP) were grown for about 20 h in a 250 mL baffled shake flask containing 25 mL of BMGY medium to an OD_{600} = 2.0. After harvesting by centrifugation, cells were resuspended in 50 mL of BSM medium in a 500 mL baffled shake flask and cultivated to the production phase. Methanol (100%) was added to a final concentration of 0.5% every 24 h to maintain induction. All procedures were performed at 28℃ and 180 rpm. At the time points 0, 24, 48, 72 and 96 h, 1 mL of expression culture

was transferred to an Eppendorf minicentrifuge tube and stored frozen at –20℃ for later analysis. *P. pastoris* strain X-33/pPICZaA (strain X-33 transformed with pPICZaA) was used as a control.

The degradation of small peptides is a common problem encountered during small peptide expression. To avoid degradation and increase the yield of the target peptide, we ensured that an optimal pH of 3.0 was maintained during the production phase (Koganesawa et al., 2002).

Purification and detection of recombinant BMP

We previously established a method to detect the target peptide BMP in beef protein hydrolyzates via high performance liquid chromatography (HPLC)–mass spectrometry/mass spectrometry (MS/MS) (Zeng et al., 2008). In previous studies, synthesized BMP was used as a standard sample and HPLC/ion trap electrospray ionization (ESI)-MS with UV detection was applied in qualitative analysis of the peptide. In the present study, this method was used to detect BMP in the fermentation broth.

Preparation of the BMP standard sample and recombinant BMP

The BMP standard sample was dissolved in double distilled water to a concentration of 10 µg/mL and was filtered through a 0.45 µm filter membrane. The filtrate was then stored at 4℃ prior to use. The fermentation broth cultures were centrifuged and the supernatant was collected. At 0, 24, 48, 72 and 96 h time points in the production phase, 1 mL of each supernatant was purified by gel-filtration chromatography using a Sephadex G-25 column (2 × 37 cm) and eluted with 0.05 mol/L NaCl solution at a flow rate of 0.4 ml/1 min. Fractions containing BMP were concentrated by lyophilization and stored at –20℃ for later analysis by HPLC-MS.

LC-MS/MS conditions

For analysis of the samples, a Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA, USA) and a Shimadzu VP ODS C_{18} analytical column (5 µm, 150 × 4.6 mm in diameter) (Shimadzu, Toyoto, Japan) was used. A sample (10 µL) was injected and the UV detection wavelength was set at 230 nm. The column was eluted at a flow rate of 0.2 mL/min in a gradient mode using a mixture of mobile phase A (H_2O + 0.1% trifluoroacetic acid) and mobile phase B (acetonitrile + 0.1% trifluoroacetic acid). The mobile phase B was increased from 5 to 50% over a 30 min period. All HPLC separations were performed at ambient temperature.
ESI/MS analysis was performed with a Surveyor-LCQ Advantage MAX instrument (Thermo Electron Corporation). Samples were automatically infused by the HPLC system. The ESI-MS/MS conditions were as follows: nitrogen was used as both the sheath gas (35 arbitrary units) and the auxiliary gas (5 arbitrary units); helium was used as the damping and collision gas; a heated capillary temperature of 300°C was used; the spray voltage was set to 4.5 kV; the collision energy was 100 V and the detection of positive ions, from 150 – 1000 u, was performed in full scan mode. Both the HPLC system and the mass spectrometer were controlled by Xcaliubur software (Thermo Electron Corporation).

RESULTS

Construction of *P. pastoris*-BMP

We designed *Xho*I and *Xba*I restriction sites to be incorporated at the ends of the PCR amplified BMP gene to allow for targeted cloning in the plasmid vector. *P. pastoris* strain X33 was transformed with the *Pme*I-linearized expression plasmid via electroporation. As shown in Figure 1, four transformants were selected on Zeocin-containing plates, designated *P.p*1, *P.p*2, *P.p*3 and *P.p*4. Then, colonies harboring multicopy plasmids were confirmed by their ability to grow on YPD plates containing increasing concentrations of Zeocin (100–2000 µg/mL).

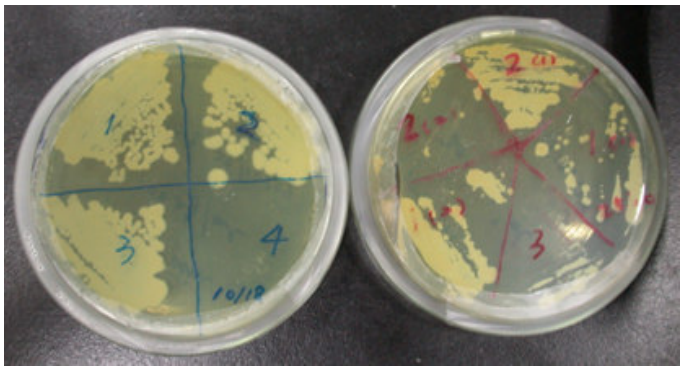

Figure 1. Screening of multiple transformants on Zeocin-containing media. Left: 100 µg/mL of Zeocin, right: 2000 µg/mL of Zeocin. 1, *P.p*1; 2, *P.p*2; 3, *P.p*3; 4, *P.p*4.

At concentrations of 100, 200, 1000 and 2000 µg/mL of Zeocin respectively, transformants *P.p*1, *P.p*2 and *P.p*3 grew well in YPD medium, but *P.p*4 did not grow at 100 µg/mL. *P.p*2 grew well in the presence of 2000 µg/mL Zeocin (Figure 1) and was therefore selected for use in subsequent experiments.

As shown in Figure 2, *P.p*2 grew better in MD medium than in MM medium. This indicated that *P.p*2 has a slow methanol utilization (Mut^S) phenotype and is unable to use methanol efficiently, highlighting the need to control the methanol concentration during fermentation.

Detection of recombinant BMP

Confirmation of BMP standard sample by HPLC and ESI-MS

Using the LC-MS conditions described above, a major peak corresponding to the BMP standard sample was obtained, with a retention time of 11.290 min (Figure 3A). In the positive ionization mode, a characteristic fragment (m/z 848.4) of the BMP standard sample was produced corresponding to the $[M-H]^+$ ion at m/z 848 (Figure 3B).

Confirmation of the recombinant BMP by HPLC and ESI-MS

LC-MS was also used to detect recombinant BMP from

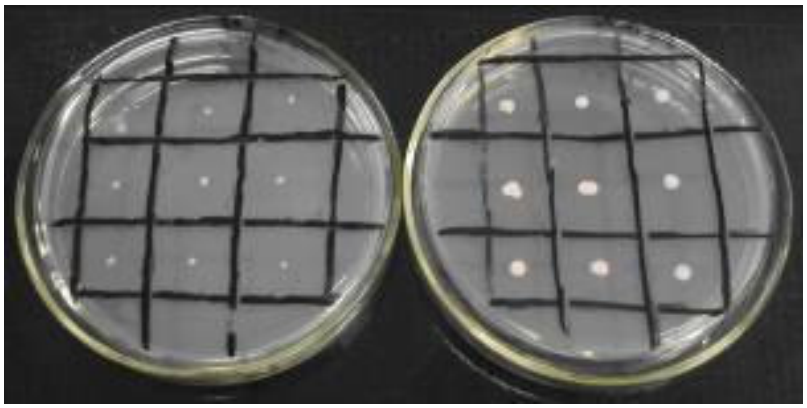

Figure 2. Determining the Mut phenotype of transformant *P.p*2. Left: MM medium, right: MD medium.

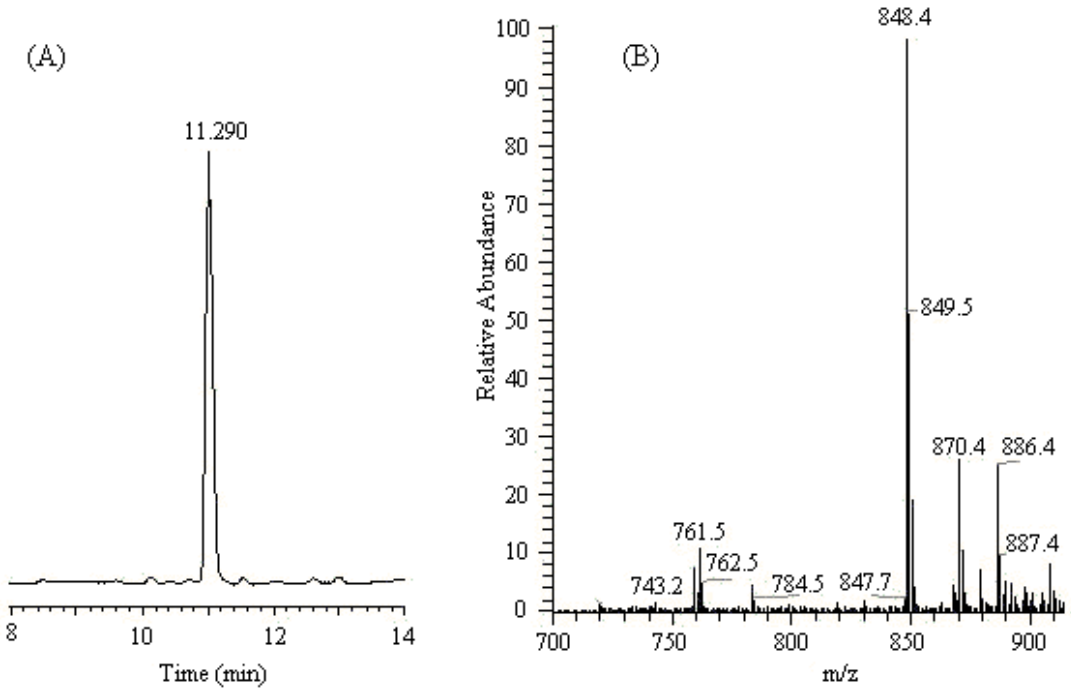

Figure 3. HPLC (A) and ESI-MS (B) analysis of the BMP standard sample.

the fermentation culture supernatant. A characteristic peak was displayed when fragments similar to those of the BMP standard sample were present. The results of ESI-MS indicated the possible presence of BMP in the fermentation culture supernatant (Figure 4) and ESI-MS/MS was carried out for further examination.

ESI-MS/MS of the BMP standard sample

ESI-MS/MS was operated in positive ion mode. The precursor ion spectra were recorded in the range of m/z 848.1 – 848.9 for the scan analysis. All data was processed using the Finnigan Xcalibur™ core data system Rev. 1.3 (Thermo Quest Corporation). According to experimental observations, as the peptides underwent collision-induced dissociation (CID), the fragments produced were predominantly b-ions (containing the N terminus) and y-ions (containing the C terminus). The nomenclature for fragmentation ions is shown in Figure 5 (Roepstorff and Fohlman, 1984).

The relative abundance of fragments is highly associated with the applied collision energy (Yamashita et al., 2007; Tsimogiannis et al., 2007; Favretto et al., 2007). The ESI-MS/MS experiments were conducted by varying the collision energy to obtain multiple characteristic

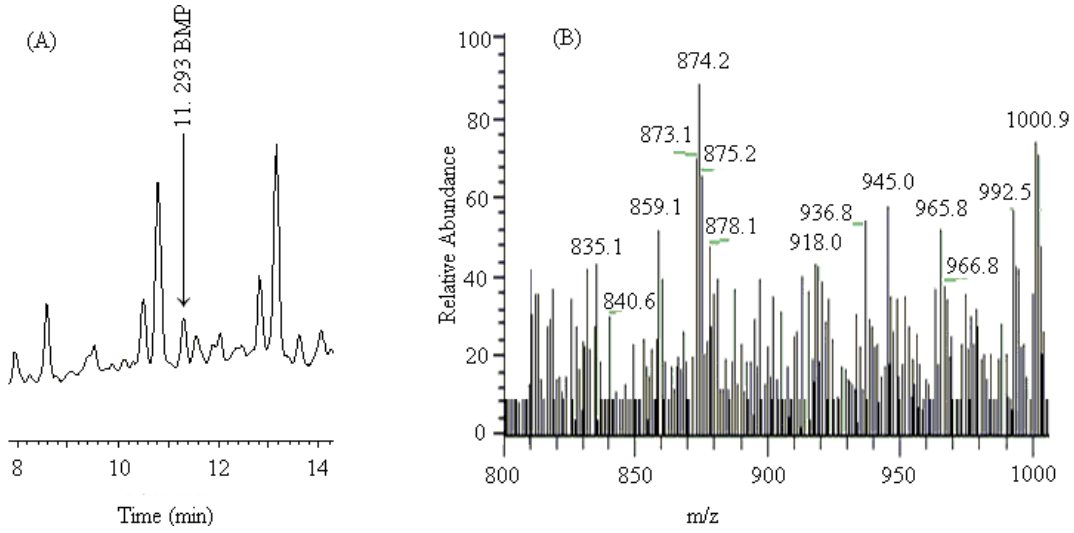

Figure 4. HPLC (A) and ESI-MS (B) analysis of recombinant BMP.

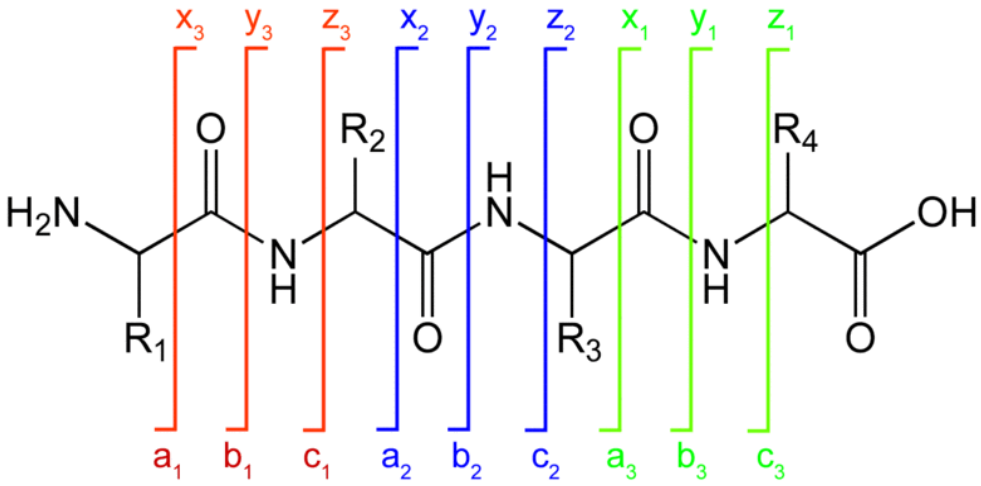

Figure 5. Sketch map of the fragment peptide backbone and the fragment nomenclature.

peaks. A series of fragment ions were obtained from in-source CID. The amino acid sequence of BMP could then be deduced from the fragment ions, that is, the b-ions and y-ions. In this study, collision energies were set at 35% for the ESI-MS/MS scans. The ESI-MS/MS spectra of the BMP standard sample are shown in Figure 6.

Fragment ions of b3, b4, b5, b6 and b7 displayed signals at m/z 301.1, 430.1, 559.2, 646.2 and 759.2, respectively. Whereas y7, $[b7\text{-}H_2O]^+$, $[x6(\text{-}CH_2\text{-}CO_2)]^+$ and $[M\text{-}H_2O]^+$ showed signals at m/z 720.1, 741.2, 631.1 and 830.2, respectively. The analysis results are listed in Table 2.

According to the ESI-MS/MS analysis conducted in this study, the sequence of the BMP standard sample was correctly identified as: Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (K-G-D-E-E-S-L-A).

ESI-MS/MS of the recombinant BMP from the culture supernatant

ESI-MS/MS analysis of recombinant BMP was performed under the same experimental conditions as those for the BMP standard sample. The ESI-MS/MS spectra of the recombinant BMP at collision energies of 35% is presented in Figure 7.

Signals for b5, b6 and b7 were detected at m/z 559.5, 646.0 and 758.8, respectively. These results showed that the peptide sequence of the recombinant BMP could be deduced from the series of fragment ions shown in Figure 7 and Table 3. Figure 7 shows that the recombinant BMP secreted from *P. pastoris* had fragment ions displaying signals at m/z 758.8 and 873.7, but not 848. However, the sequence of the first seven amino acids (Lys-Gly-

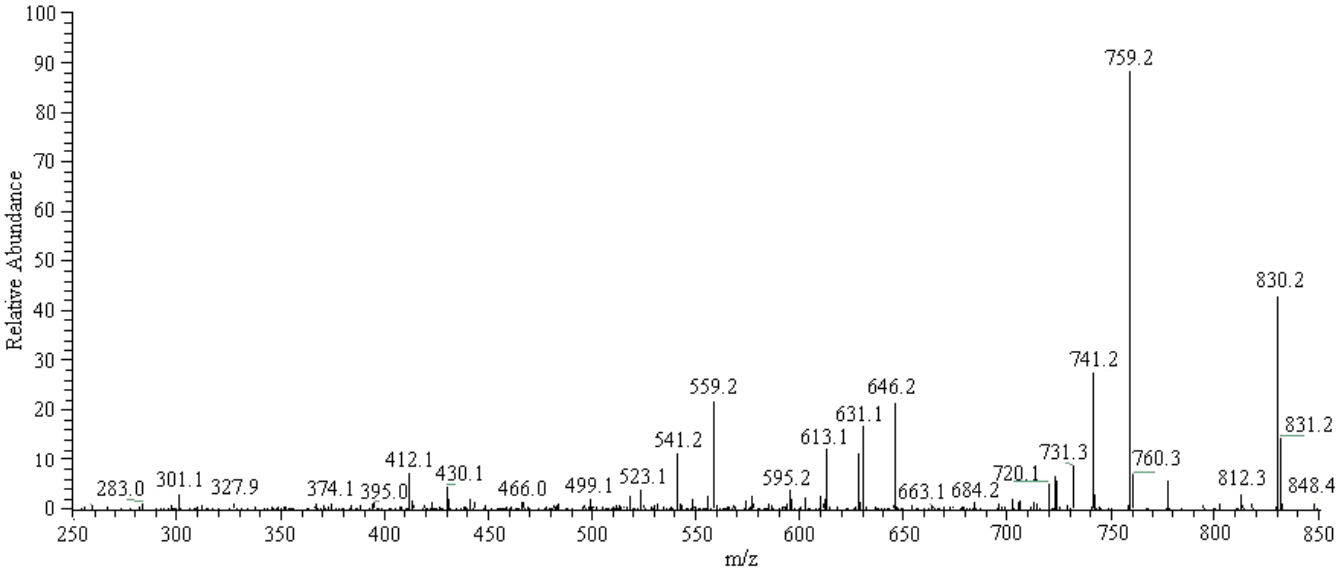

Figure 6. ESI-MS/MS analysis of the BMP standard sample.

Table 2. Fragment ions of the BMP standard sample, as determined by mass spectrometry.

Fragment ion	Sequence	Expected mass (m/z)	Measured mass (m/z)
b1	K	129	------
b2	KG	186	------
b3	KGD	301	301.1
b4	KGDE	430	430.1
b5	KGDEE	559	559.2
b6	KGDEES	646	646.2
b7	KGDEESL	759	759.2
y7	GDEESLA	720	720.1
$[b7\text{-}H_2O]^+$	KGDEESL	741	741.2
$[x6(\text{-}CH_2\text{-}CO_2)]^+$	GDEESL	631	631.1
$[M\text{-}H_2O]^+$	KGDEESLA	830	830.2

Asp-Glu-Glu-Ser-Leu) in the recombinant BMP sequence was found to be identical to that of the BMP standard sample by ESI-MS/MS analysis. By comparing the spectra of the peaks at 873.7 and 758.8, we speculated that the last amino acid, Ala, of the BMP sequence was replaced with Pro (with a relative molecular weight of 115 Da) in the BMP fragment. This would result in the primarystructure of recombinant BMP being: H-Lys-Gly-Asp-Glu-Glu-Ser-Leu-Pro-OH (K-G-D-E-E-S-L-P). Future studies should investigate whether this change could lead to deactivation, and the mechanism of replacement remains to be elucidated.

Production of recombinant BMP

The yield of BMP was estimated from the reverse phase (RP)-HPLC data, using the areas under the peaks. The fermentation time is reported to be important for the expression of BMP (Kerry-Williams et al., 1998) and the optimal inducing time was investigated in this study. In the initial 48 h induction, BMP was undetectable in the fermentation culture, indicating that the *P.p*2 transformant is Mut^S and cannot utilize methanol efficiently. After the addition of methanol to induce BMP expression, it may take the cells some time to adapt to the high concentration of methanol and then begin to secrete and accumulate BMP. After 72 h of induction, the final yield of purified recombinant BMP from each liter of culture broth was as high as 10 mg.

DISCUSSION

The small tasty recombinant peptide BMP was expressed in *E. coli* or *S. cerevisiae* host cells, but the yield of BMP

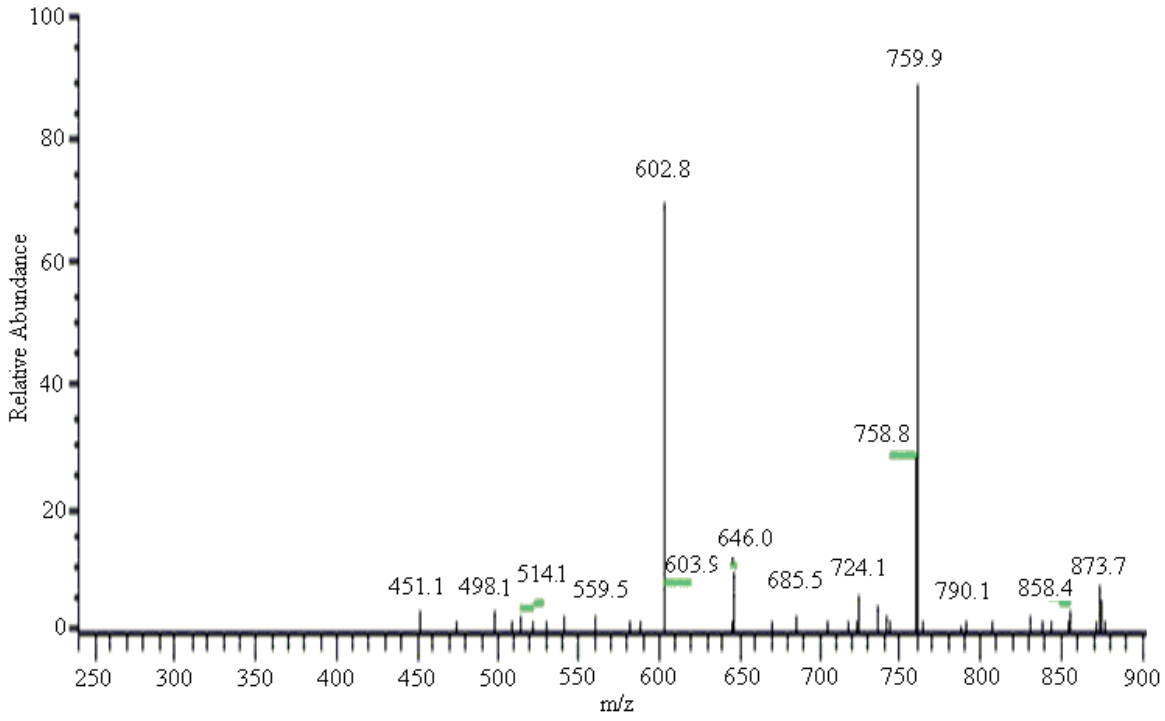

Figure 7. ESI-MS/MS analysis of recombinant BMP.

Table 3. Fragment ions of recombinant BMP, as determined by mass spectrometry.

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b1	K	129	------
b2	KG	186	------
b3	KGD	301	------
b4	KGDE	430	------
b5	KGDEE	559	559.5
b6	KGDEES	646	646.0
b7	KGDEESL	759	758.8

was not reported in the patent literature (Lerch et al., 1997). To our knowledge, the production levels of the foreign soluble, secretable proteins in these expression system are often less than desirable (Macauley-Patrick et al., 2005).

Compared with *E. coli* or *S. cerevisiae*, the *P. pastoris* is being increasing employed as an efficient host for secretory expression of heterologous proteins due to several important advantages.

One advantage is that it has ease of genetic manipulation, e.g. gene targeting, high-frequency DNA transformation, cloning by functional complementation, high levels of heterologous proteins expression in this host at the intracellular or extracellular level (Macauley-Patrick et al., 2005; Cereghino et al., 2002; Cregg et al., 2000; Schipperus et al., 2009). A second important advantage is that *P. pastoris* can be grown to unusually high-cell densities (> 100 g/L dry cell weight) in simple minimal salt medium, and scale-up of *P. pastoris* fed-batch cultures is relatively easy. A third advantage is that *P. pastoris* secrets only low levels of its own proteins, thus, heterologous secreted proteins are often the predominant protein found in the culture medium which facilitates protein purification (Koganesawa et al., 2002). Therefore, the powerful genetic techniques available, together with its economy of use, make *P. pastoris* a system of choice for heterologous protein expression (Macauley-Patrick et al., 2005; Cregg and Sunga, 2004; Koganesawa et al., 2002).

Here, we report the production of BMP in the methylotrophic yeast *P. pastoris*. In this study, the phenotype of the transformant *P.p2* was found to be Mut^S. This transformant was unable to utilize methanol effectively and BMP production was low. In most cases,

Mut$^+$ strains are selected as production strains because they can depend solely on methanol as the carbon source and can adapt to new environments containing methanol. The expression of BMP is regulated by the *aox* promoter and this promoter is induced by methanol. So Mut$^+$ strains are generally more suitable for expression of heterologous proteins. In future studies, Mut$^+$ mutants could be selected to potentially enhance the production of BMP.

We predicted that degradation of small peptides may be an important problem in the expression process. During the expression of heterologous peptide, many proteases are also secreted into the fermentation culture which may subsequently promote the degradation of the target peptide. There are many factors that affect the activity of proteases and the most important of these is pH (Inan et al., 1999; Kobayashi et al., 2000; Koganesawa et al., 2002; Jahic et al., 2003). In our experiments, no BMP was detected by RP-HPLC at pH 5.0 or 6.0, but BMP was detected at pH 3.0 indicating that a lower pH may inhibit small peptide degradation. There are other methods of reducing peptide degradation, such as the addition of nitrogen. It was reported that inadequate nitrogen concentrations promote the activity of proteinases and that the addition of yeast extract or amino acids could greatly decrease this degradation and enhance the expression of small peptides (Kobayashi et al., 2000). Maintaining a low temperature during fermentation is another effective method, as this also inhibits the activity of proteinases (Takahashi et al., 2001; Sreekrishna et al., 1997). These methods could be applied to the production of BMP in future studies to control peptide degradation.

Recombinant DNA technology has many potentially promising applications in the discovery and production more cost-effective, novel agents in the flavor and fragrance industry. Furthermore, this technology may be instrumental in advancing our understanding of olfaction and taste, which will in turn lead to the discovery of novel odor- and taste-modifying compounds, changing the way flavor and fragrance compositions will be formulated in the future.

To obtain a plentiful supply of BMP and minimize the cost of the product, strategies must be developed to overexpress this small tasty peptide on a large scale, producing this peptide as efficiently as possible. One promising approach may be to construct *P. pastoris* strains harboring tandem repeats of the BMP gene in their genome, as these multicopy strains often display high expression levels (Yan at al., 2003; Koganesawa at al., 2002).

ACKNOWLEDGMENTS

This work was financially supported by Tianjin Municipal Development of Science and Technology, China (Project No. 05YFGHHZ00200).

REFERENCES

Bolen PL, Cihak PL, Scharpf, LG JR, Miller KP, Kossiakoff N, Hawn RD (2003). Flavor active modified thaumatin and monellin and methods for their production and use. United States Patent Application: USP20030050445.

Cereghino GPL, Cereghino JL, Ilgen C, Cregg JM (2002). Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Curr. Opin. Biotechnol., 13: 329-332.

Cregg J, Cereghino J, Shi J, Higgins D (2000). Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol., 16: 23–52.

Cregg JM, Sunga AJ (2004). The *Pichia pastoris* formaldehyde dehydrogenase gene (*FLD1*) as a marker for selection of multicopy expression strains of *P. pastoris*. Gene, 330: 39-47.

Favretto D, Frison G, Maietti S, Ferrara SD (2007). LC-ESI-MS/MS on an ion trap for the determination of LSD, *iso*-LSD, *nor*-LSD and 2-oxo-3-hydroxy-LSD in blood, urine and vitreous humor. Int. J. Legal Med., 121: 259-265.

Inan M, Chiruvolu V, Eskridge KM, Vlasuk GP, Dickerson K, Brown S, Meagher MM (1999). Optimization of temperature–glycerol–pH conditions for a fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*. Enzyme Microb. Tech., 24(7): 438-444.

Invitrogen (2010). EasySelect *Pichia* Expression Kit. For Expression of Recombinant Proteins Using pPICZ and pPICZα in *Pichia pastoris*. Cat. no. K1740-01. Invitrogen.

Jahic M, Gustavsson M, Jansen AK, Martinelle M, Enfors SO (2003). Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes. J. Biotechnol., 102(1): 45-53.

Kerry-Williams SM, Gilbert SC, Evans LR, Balance DJ (1998). Disruption of the *Saccharomyces cerevisiae YAP3* gene reduces the proteolytic degradation of secreted recombinant human albumin. Yeast, 14(2): 161-169.

Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Ohi H, Tomomitsu K, Ohmura T (2000). High-level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. J. Biosci. Bioeng., 89(1): 55-61.

Koganesawa N, Aizawa T, Shimojo H, Miura K, Ohnishi A, Demura M, Hayakawa Y, Nitta K, Kawano K (2002). Expression and purification of a small cytokine growth-blocking peptide from armyworm *Pseudaletia separata* by an optimized fermentation method using the methylotrophic yeast *Pichia pastoris*. Protein Expres. Purif., 25: 416-425.

Lerch K, Muheim A, Silke N (1997). Cloning, expression and production of tasty peptides. European Patent Application: EP0832972A2.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005). Heterologous protein production using the *Pichia pastoris* expression system. Yeast, 22: 249-270.

Muheim A, Hausler A, Schilling B, Lerch K (1998). The impact of recombinant DNA technology on the flavor and fragrance industry. Perfumer Flavorist, 23: 21-27.

Roepstorff P, Fohlman J (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed. Mass Spectrom., 11(11): 601.

Schipperus R, Teeuwen RLM, Werten MWT, Eggink G, de Wolf FA (2009). Secreted production of an elastin-like polypeptide by *Pichia pastoris*. Appl. Microbiol. Biotechnol., 85: 293–301.

Sreekrishna K, Brankamp RG, Kropp KE, Blankenship DT, Tsay JT, Smith PL, Wierschke JD, Subramaniam A, Birkenberger LA (1997). Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene, 190(1): 55-62.

Takahashi K, Takai T, Yasuhara T, Yokota T, Okumura Y (2001). Effects of site-directed mutagenesis in the cysteine residues and the N-glycosylation motif in recombinant Der f 1 on secretion and protease activity. Int Arch Allergy Immunol., 124(4): 454-460.

Tsimogiannis D, Samiotaki M, Panayotou G, Oreopoulou V (2007). Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS. Molecules, 12: 593-606.

Wang K, Maga JA, Bechtel PJ (1995). Stability of beefy meaty peptide to pasteurization and sterilization temperature. Lebensm.-Wiss. u.-

Technol., 28: 539-542.
Wang K, Maga JA, Bechtel PJ (1996). Taste properties and synergisms of beefy meaty peptide. J. Food Sci., 61(4): 837-839.
Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S, Numazawa M (2007). Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry. Steroids, 72: 819-827.
Yamasaki Y, Maekawa K (1978). A peptide with delicious taste. Agric. Biol. Chem., 42: 1761-1765.
Yamasaki Y, Maekawa K (1980). Synthesis of a peptide with delicious taste. Agric. Biol. Chem., 44: 93-97.
Yan YJ, Chen JC, Li J (2003). Overexpression of a small medicinal peptide from ginseng in the yeast *Pichia pastoris*. Protein Expres. Purif., 29: 161-166.
Zeng SR, Wang YP, Bai XJ, Lin WL, Yang M, Xing HP (2008). Determination of meaty peptide in enzymatic hydrolyzate of beef Protein by HPLC-MS. Korean J. Chem. Eng., 25(5): 1120-1124.